Lipid Composition of Cyst Stages of *Globodera rostochiensis*

Globodera rostochiensis (golden cyst nematode) has an intimate relationship with its host plant (*Solanum tuberosum* and related species of potato) in which lipids play an important part. The lipid layer of the eggshell of *G. rostochiensis* provides a barrier from desiccation (20) and appears to play a key role in hatching in response to potato root diffusate (9,22). After hatching, lipid reserves are important to survival of nonfeeding stages of various plant-parasitic nematodes, and lipid reserve depletion is correlated with reduced infectivity, reduced motility, and delayed development in juveniles (24,25,29,30). Once the female is attached to a feeding site on the plant root cell membrane, both neutral and polar lipids are incorporated into the developing eggs. Parasitic stages likely require lipids or lipid precursors, such as sterols and fatty acids, in the diet for normal development, which are at least partly supplied by the host plant (3,15).

Although some literature is available on the lipid profile of plant-parasitic nematodes (4,5,7,15,16,20), most studies on nematode lipids have been done on microbivorous or animal-parasitic forms (3,6,8,26–28,33) and have concentrated on total lipids, neutral lipids, and fatty acid profiles of adult nematodes. This work was undertaken to provide a complete lipid analysis of the white, yellow, and brown cyst stages of *G. rostochiensis*.

Materials and Methods

Chemicals: Lipid class and fatty acid standards were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were reagent grade except for solvents, which were HPLC grade (Fisher Scientific, Pittsburgh, PA).

Cyst production and collection: Cysts of *G. rostochiensis* were produced on pot-grown potato plants under a 14-hour photoperiod in a greenhouse. Potato *(S. tuberosum)* seed pieces 3.5–5.0 cm in diameter were planted in 7.5-cm-d clay pots filled with a 1:1 mixture of steam sterilized soil and sand and inoculated with 5,000 viable eggs/pot. Starting at 5 weeks after planting, plants were visually assessed for cyst

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numbers on the root mass. Potted plants with at least 50 cysts/pot were set aside for cyst collection during the winters of 1993 and 1994. The stem of each plant was cut 5 cm above the soil surface and the root mass was removed intact. Cysts were collected from the outside surface of roots by a slight touch of a prewetted scalpel, then placed in distilled water until a sufficient number (approximately 50–100 mg/individual sample size) were collected. Cysts were separated manually into white, yellow, and brown stages during harvest. A subsample of cysts from each stage was examined for the presence of fully developed second-stage juveniles as a measure of maturity. Between 10 (brown) to 50 (white and yellow) cysts were examined for each stage in order to define the range of maturity within each stage.

Cysts were resuspended, with several transfers, in distilled water to remove any traces of soil. Excess water was removed via pipette, and cyst samples (50–100 mg/sample) were transferred with a spatula to a tared vial containing 2 ml of boiling isopropanol in a dry thermal bath. Each cyst sample was then boiled for 20 minutes to inactivate lipolytic enzymes, adding additional boiling isopropanol as needed. Cyst collection and treatment with isopropanol were carried out within a 6-hour time period for each harvest. Preliminary experiments using brown cysts directly ground in chloroform:methanol:H_2O (1:2:0.8) produced a lipid extract consisting mostly of free fatty acids with only minor amounts of phospholipids and neutral lipid, suggesting enzymatic hydrolysis during extraction. Upon inclusion of a boiling isopropanol step following cyst isolation, only low levels of free fatty acids were found in various cyst samples. Vials were then stored at −20 C before lipid extraction.

Lipid extraction: A slight modification of the procedure of Bligh and Dyer (1) was used. Excess isopropanol was removed by evaporation under nitrogen using a Pierce reacti-therm module (Pierce Chemical, Rockford, IL) set at 50 C. After samples were dried and weighed, cysts were transferred to a ground glass homogenizer in 0.5 ml of chloroform:methanol:H_2O (1:2:0.8), and an additional 1.5 ml was added. After homogenization, the extract was filtered through Whatman No. 1 paper on a Büchner funnel, rinsed with an additional 0.5 ml of chloroform:methanol:H_2O (1:2:0.8), and placed into a glass test tube fitted with a Teflon-lined cap. Water and chloroform (2 ml of each) were added to the test tubes, mixed, and then allowed to partition into two layers. In some cases, tubes were centrifuged at 1,000*g* for 10 minutes to facilitate separation of the layers with a clear interface. The chloroform layer was removed to a tared tube and then evaporated to dryness in a Savant rotary vacuum concentrator. After weights were taken, the sample was resuspended in chloroform:methanol (1:1), filtered through a 0.4-μm PVDF filter, and analyzed. For each cyst stage, at least five replicates were prepared for analysis from each of the winter 1993 and 1994 periods.

Lipid class analysis: Lipid classes were analyzed by high performance liquid chromatography (HPLC) (18). Briefly, this HPLC method employs a silica column and an iso-octane-isopropanol-water solvent system, with a flame ionization detector (FID) to quantify lipid classes and a UV detector set at 205 nm to assess degree of unsaturation present in each lipid class. Peaks were identified by comparing retention times with known lipid standards. Duplicate injections of two independent sets of pooled samples (2–3 individual lipid extracts) were analyzed for each cyst category. The composition of total lipids was calculated for an individual lipid class by integration of the HPLC peak area and expression as μg of each lipid class per mg total lipid ± SD.

Fatty acid analysis: For each cyst stage, extracts from 4–6 cyst samples of white, yellow, and brown cyst preparations were pooled in order to obtain sufficient material (2–5 mg lipid) for fatty acid analysis. The total lipid samples were hydrolyzed with methanolic KOH and the resulting free fatty acids were converted to methyl

esters with diazomethane as previously described (17). Fatty acid methyl esters were separated and quantified with a Hewlett-Packard 5890 gas chromatograph equipped with on-column injection, FID, and a 30-m × 0.32-mm DB-225 polar capillary column (J & W Folsom, CA). The temperature program started at 100 C, increasing linearly to 180 C at a rate of 20 degrees/minute, then linearly to 220 C at 5 degrees/minute, followed by a 14.5-minute hold at 220 C. The fatty acids were identified and quantified by comparing retention times and peak areas with those of authentic standards. Duplicate injections of a pooled sample at each cyst stage were conducted and mean ± SD determined as a percentage of total fatty acid.

Results

Cyst characterization: Maturity was defined as the percentage of the population within a cyst represented by fully developed second-stage juveniles. The white stage consisted mainly of small cysts containing undeveloped eggs (Table 1). The yellow cyst preparations consisted of a mixture of cysts ranging from a color gradation of a yellow tint to those with a strong yellow color; this collection period had the largest variability in egg maturity. The brown cyst preparations were more uniform in both coloration and maturity stage.

Total lipids: Lipid levels, as measured by $CHCl_3$ soluble material, were 4.2% g fresh weight (±1.4 S.E.) in the white (gravid female) cyst stage, 4.8% g fresh weight (±0.6 S.E.) in the yellow cyst stage, and 2.0% g fresh weight (±0.2 S.E.) in the brown cyst stage. The actual percentages are not absolute values because the processing method required to minimize lipid hydrolysis (fresh weights taken following a boiling isopropanol step) may have introduced sampling error.

Table 1. Egg maturation levels of *Globodera rostochiensis* cysts.

Stage	Weeks after inoculation	Maturity (%)
White	5–6	0
Yellow	6–8	10–40
Brown	9–11	80–100

Lipid class analysis: A complex mixture of lipid classes was identified by HPLC in all cyst preparations (Fig. 1). Chromatograms from replicate samples in the white, yellow, and brown cyst preparations indicated similar classes of lipids were present in all preparations. With both the choline phosphoglycerides and ethanolamine phosphoglycerides peaks, the ratio of the UV peak area to the FID peak area was greater than that observed with plant lipid extracts, suggesting a high degree of phospholipid unsaturation. In some of the preparations, the UV chromatogram indicated two or more components within the ethanolamine phosphoglyceride and choline phosphoglyceride fractions.

The triacylglycerol fraction was approximately 69%, 75%, and 54% of the total lipid in the white, yellow, and brown cyst stages, respectively (Table 2). Brown cyst preparations contained the highest levels of free fatty acids (21% of total lipid versus 2% in yellow and white cysts). The percentage of free fatty acids + triacylglycerol fractions (75%) in brown cysts approximated the triacylglycerol levels found in white and yellow cysts.

Both ethanolamine phosphoglycerides and choline phosphoglycerides were present in high amounts in all cyst stages (Table 2). The total phospholipid fraction was approximately 21%, 15%, and 13% in the white, yellow, and brown cyst stages, respectively. The ethanolamine phosphoglycerides fraction decreased only slightly (16%) from the white to the brown stages, while the choline phosphoglycerides fraction decreased approximately 50% from the white to the brown cyst stage.

Several minor lipid classes also differed quantitatively among the three cyst stages. The proportion of lipid in the steryl ester fraction increased 2.8-fold from the white to the brown cyst stage, while free sterols remained relatively unchanged at all

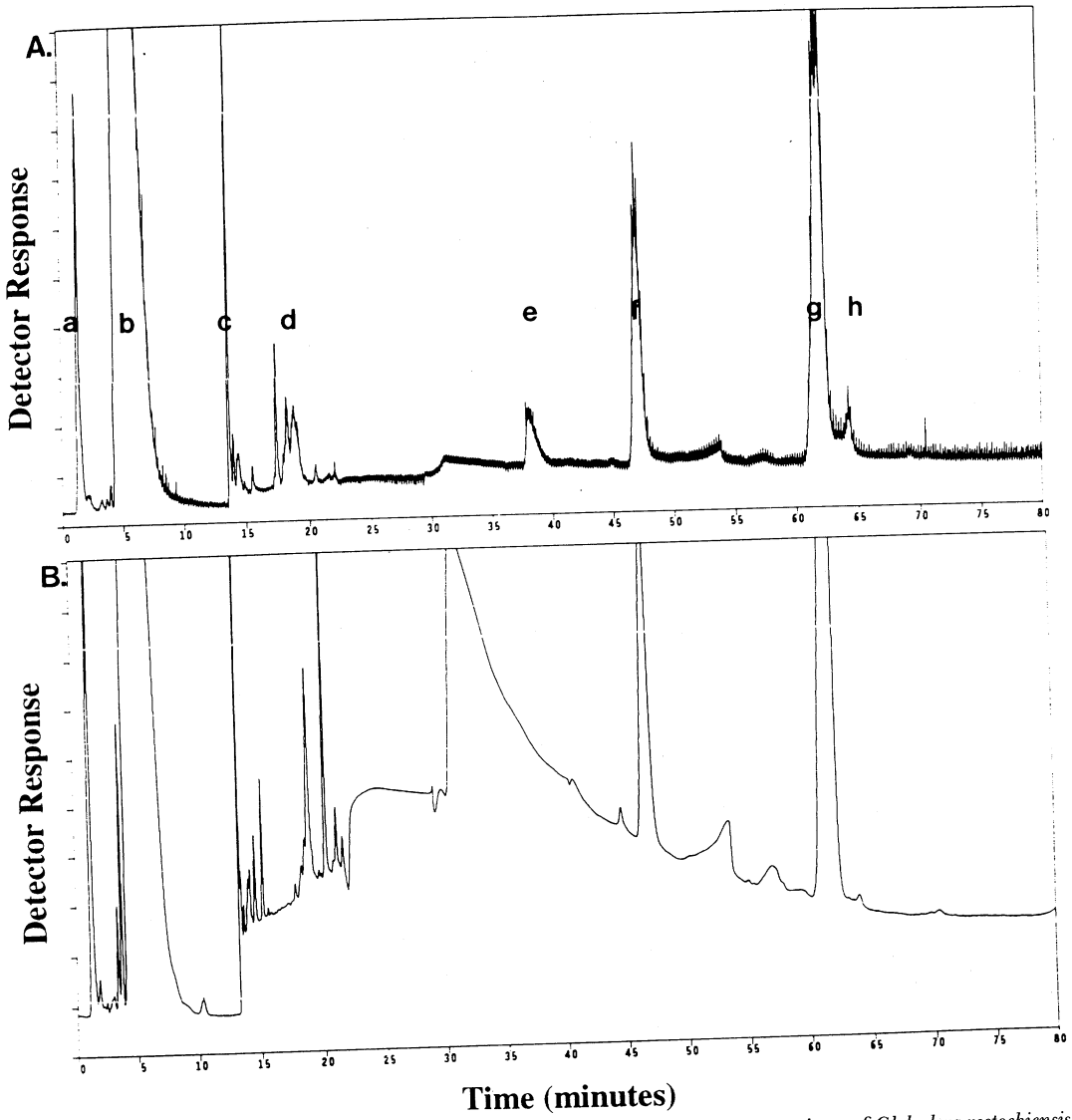

FIG. 1. Typical chromatogram of lipid classes in extracts of yellow cyst preparations of *Globodera rostochiensis* obtained by HPLC using both (A) flame ionization and (B) UV (205-nm) detectors. In the elution profile, nonpolar lipids elute early and polar lipids elute later. The lettered peaks have retention times corresponding to the following lipid standards: (a) steryl ester; (b) triacylglycerol; (c) free sterols; (d) free fatty acids; (e) cardiolipin; (f) ethanolamine phosphoglycerides; (g) choline phosphoglycerides; (h) sphingomyelin.

stages. The sphingomyelin fraction approximately doubled from the white to the brown stage, while the cardiolipin fraction was observed only in the white and yellow cyst stages.

Fatty acid analysis: The fatty acid compositions among the cyst stages were quite similar (Table 3). Eicosenoic acid (20:1) and eicosatetraenoic acid (20:4), identified as arachidonic acid, were found in high concentrations in all cyst preparations and accounted for approximately 45% of the total fatty acids present. Octadecenoic acid (18:1), identified as vaccenic acid, was the third most abundant fatty acid found in all three cyst samples. The identity of both vaccenic and arachidonic acid was confirmed with authentic standards, based on retention times.

The fatty acid profiles contained a high proportion of unsaturated fatty acids (ca. 78% of total). In addition, 60% of the fatty

TABLE 2. Lipid classes in three developmental stages of *Globodera rostochiensis* cysts.

Lipid	White	Yellow	Brown
Steryl esters	27.4 ± 0.4	23.3 ± 2.1	77.8 ± 2.1
Triacylglycerols	697.7 ± 34.2	748.2 ± 27.6	542.3 ± 20.9
Sterols, free	45.2 ± 11.3	35.3 ± 9.6	27.9 ± 6.2
Free fatty acids	21.8 ± 4.0	20.1 ± 2.2	211.8 ± 10.3
Cardiolipin	8.8 ± 0.1	12.5 ± 0.3	trace
Ethanolamine phosphoglycerides	60.7 ± 2.4	48.1 ± 0.4	50.9 ± 0.5
Choline phosphoglycerides	151.5 ± 5.9	99.2 ± 1.0	77.5 ± 0.8
Sphingomyelin	5.5 ± 0.7	13.2 ± 3.7	12.7 ± 0.7

Results are expressed as μg/mg ± SD of total lipid, based on HPLC flame ionization detection.

TABLE 3. Fatty acid composition of three developmental stages of *Globodera rostochiensis* cysts, expressed as relative percentage of total fatty acid.

Fatty acid	Weight % ± SD		
	White	Yellow	Brown
C16:0	1.88 ± 0.02	1.98 ± 0.01	2.74 ± 0.01
C16:1 Δ9	0.44 ± 0.05	0.52 ± 0.01	0.65 ± 0.02
C18:0	7.97 ± 0.01	7.83 ± 0.10	8.73 ± 0.02
C18:1Δ9	2.68 ± 0.05	2.35 ± 0.01	2.36 ± 0.06
C18:1 Δ11	13.31 ± 0.06	12.41 ± 0.11	13.89 ± 0.02
C18:2 Δ9, 12	2.63 ± 0.23	2.63 ± 0.01	2.78 ± 0.06
C18:3 Δ6,9,12	3.41 ± 0.31	3.42 ± 0.04	2.20 ± 0.04
C20:0	5.15 ± 0.11	5.08 ± 0.04	6.61 ± 0.05
C20:1 Δ11	22.19 ± 0.61	22.95 ± 0.04	24.85 ± 0.03
C20:2Δ11,14	1.31 ± 0.11	1.47 ± 0.07	1.30 ± 0.11
C20:3 Δ11,14,17	10.16 ± 0.02	8.95 ± 0.02	6.40 ± 0.24
C20:4 Δ5,8,11,14	20.93 ± 0.08	23.33 ± 0.36	21.25 ± 0.04
C20:5 Δ5,8,11,14,17	trace	0.25 ± 0.03	trace
C22:0	1.99 ± 0.01	1.84 ± 0.04	1.82 ± 0.16
C22:1 Δ13	0.63 ± 0.04	0.51 ± 0.02	0.47 ± 0.01
other	5.83 ± 0.48	4.08 ± 0.30	4.20 ± 0.02

acids present were C20 or longer in all preparations.

DISCUSSION

Total lipid contents have previously been shown to vary widely from one nematode species to another, perhaps dependent upon the habitat and developmental stage of the nematode. For example, total lipid levels on a dry-weight basis was 10–11% *(Aphelenchoides ritzemabosi)*, 25–28% *(Pratylenchus penetrans)*, and 37–38% *(Ditylenchus dipsaci)* (15); other studies have found similar variations among other nematode species (4–7,19,33). Adult females of *Meloidogyne incognita* and *M. arenaria* contained 46% and 40% lipid, while eggs contained 64% and 67% lipid on a dry-weight basis (16). In our study, only fresh weights were measured. If a rough estimate of approximately 50% water is assumed in these cyst preparations, the total lipid content reported here would be in the range of 4–10% total lipid on a dry-weight basis. Because the goal of this study was to focus on lipid components, the samples were not subjected to exhaustive extraction, and the total lipids present may have been greater than the 4–10% of cyst dry weight.

Our studies indicate that neutral lipids are the major component of total lipids in eggs and juveniles of *G. rostochiensis* (54–75% of total lipid), confirming data obtained previously by scanning microdensitometry (24,25,29,30). We have further identified the major class of neutral lipids present as the triacylglycerol fraction. De-

teriorated female tissues might account for the lower triacylglycerol content and subsequent 10-fold higher free fatty acid content of the brown cysts relative to the white and yellow preparations. The triacylglycerol fraction of *G. solanacearum* females, similarly, comprised 50% of total lipid in cysts of that species (19). In another cyst nematode, *Heterodera zeae,* the triglycerides comprised 74% of total lipid and 85% of the neutral lipid fraction (4). The high amount of triacylglycerols we measured would likely provide the major energy reserve of the second-stage juvenile, allowing it to survive for prolonged periods of time after hatching (23–25).

Polar lipids in females and eggs of *Meloidogyne incognita* and *M. arenaria* were reported to be 6–8% of total lipid (16). In *G. rostochiensis* cysts, we found polar lipids at higher concentrations relative to total lipid (20–26%). The amount of phospholipids in cysts (13–20% of total lipid) was somewhat higher than that reported recently for the free-living nematode *Caenorhabditis elegans* (187 ± 46.7 μg/g fresh weight) (28). The relative proportion of phospholipid classes also differs; the bulk (60–72%) of phospholipid in *G. rostochiensis* cysts is present as choline phosphoglycerides, and most of the phospholipid in *C. elegans* (54.5%) is present as ethanolamine phosphoglycerides. Previously, however, in both *Turbatrix aceti* and *Meloidogyne javanica* preparations, choline phosphoglycerides were the major phospholipid class followed by ethanolamine phosphoglycerides (5,6), similar to levels observed in this study. We also observed multiple peaks within the ethanolamine phosphoglyceride and choline phosphoglyceride peaks, which may indicate a small degree of separation of molecular species within these lipid classes. Alternatively, these multiple peaks might also indicate the presence of ether lipids or plasmalogens.

Phospholipids are the major lipid constituent of membranes. In *G. rostochiensis* and *G. pallida,* the eggshell consists of an outer vitelline layer, a chitinous layer, and an inner lipid layer, which changes from a semipermeable to a permeable state when stimulated by potato root diffusate (9,12). The inner lipid layer of *G. rostochiensis* is composed of a variable number of lipoprotein membranes (usually 2–3 membranes) (22). The high concentration of phospholipid we measured in *G. rostochiensis* cysts may be due to the lipoprotein membranes surrounding eggs within cysts (22).

The high degree of unsaturation present in the fatty acid profiles of cyst preparations of *G. rostochiensis* is consistent with adaptation to cold temperatures by invertebrates (14). Frost is common among the high elevations of the Andean region where *G. rostochiensis* is indigenous, and *G. rostochiensis* has spread to many regions of the world where winter soil temperatures are below freezing. A high proportion of unsaturated fatty acids in phospholipids generally increases the fluidity of membrane structures at low temperatures, which would provide cold protection by preserving membrane integrity. Unhatched second-stage *G. rostochiensis* juveniles have been reported to survive exposure to subzero temperatures in the presence of water, whereas hatched juveniles were susceptible (21). A recent report noted a high degree of unsaturation of lipid, indicated by broad spectral peaks, in hydrated cysts of *Globodera pallida* (a potato cyst nematode closely related to *G. rostochiensis*) using the noninvasive technique of ^{13}C NMR spectroscopy (13). We were able to provide quantitative evidence for a high degree of unsaturated fatty acids in *G. rostochiensis* as well, with unsaturated fatty acids accounting for more than 75% of total fatty acids present.

The fatty acid profile obtained in our study contains a high degree of unsaturated fatty acids (78%), with 60% as C-20 or longer, and a relatively broad array of fatty acids present. The three predominant fatty acids were vaccenic (18:1), eicosenoic (20:1), and arachidonic (20:4). Our levels are higher than those reported for *G. solanacearum* females (60% unsaturated), but were similar in profile (19). Vaccenic acid is also found as the major fatty acid in fe-

males and eggs of *M. incognita* and *M. arenaria* (16), and eicosenoic acid has been reported to be present in high concentrations in several invertebrates (31,32).

Arachidonic and eicosapentaenoic acids are known to be phytoalexin elicitors for potato (2,11), stimulating the release of rishitin and lubimin in potato tuber tissue. Both fatty acids are found in mycelial extracts of *Phytophthora infestans,* the fungal pathogen that causes late blight of potatoes (10,11) and are implicated in the hypersensitive response that occurs when potato is infected (2,11). Their presence in *G. rostochiensis* suggests that they may also play a role in the interaction of this potato pathogen with its host plant.

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